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The chemical analysis of small samples of leaf material and the relationship between the disappearance and composition of leaves

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Synopsis

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Beech leaves (*Fagus silvatica* L.) collected at leaf-fall were arbitrarily divided into "hard" and "soft" categories. During exposure to decomposition processes in the litter layer of beech woodland for up to 4 months, samples were taken, ground up and the ash, sugars, lignin, polyphenols, total nitrogen and methanol and petroleum-ether soluble fractions assessed by a scheme of analysis able to deal with only one hundred milligrammes of leaf material. Results indicate that amount of polyphenol found was inversely proportional to the rate at which leaves disappeared, and that hard leaves had more polyphenolic substances than soft ones.

Nach dem Blattfall gesammelte Buchenblätter (von *Fagus silvatica* L.) wurden willkürlich in Gruppen von „harten“ und „weichen“ Blättern geschieden.

Während die Blätter in der Streuschicht eines Buchenwaldes bis zu 4 Monaten den natürlichen Abbauprozessen ausgesetzt waren, wurden Proben entnommen und fein zermahlen. Von diesen Proben wurde der Gehalt an Asche, Zucker, Lignin, Polyphenolen, Gesamt-Stickstoff und methanol- bzw. petrolätherlöslichen Fraktionen nach einem Analysenschema bestimmt, das geeignet ist, mit nur 100 mg Blattsubstanz zu arbeiten. Die Ergebnisse weisen darauf hin, daß die Menge des gefundenen Polyphenols der Menge der abgebauten Blattsubstanz umgekehrt proportional war und daß harte Blätter mehr polyphenolische Substanzen beinhalten als weiche.

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1. Introduction

HEATH, EDWARDS and ARNOLD (1964a) estimated visually and photometrically the amount of leaf litter eaten by soil fauna. Their observations need to be supplemented by information about the chemical composition of the litter, and this paper describes a scheme of analysis, suitable for 0.5—1.0 g of leaf material, for assessing properties that possibly influence palatability, for example, texture, nutritional value and astringency. Several different successive determinations can be made on a leaf sample, even when only a few hundred milligrams remain from a feeding experiment; the sequence is arranged so that the most information is gained from the least material.

Leaf texture probably depends on structural materials, particularly lignin, and on the thickness of the waxy outer coating, which would be expected to affect attack by small animals, and hence influence the rate of decomposition. The palatability of a leaf is probably influenced by its content of polyphenols and sugars. Because thick hard leaves

disappear more slowly than soft leaves, HEATH (1962) suggested that toughness influences palatability.

It seems that many constituents affect leaf litter disappearance:

- a) Polyphenols related to the rate of disappearance of oak leaf discs (HEATH and KING, 1964);
- b) Protein content determined as total N, important in changes in decomposing leaf litter (SOKOLOV, 1960; GILBERT and BOCK, 1960);
- c) Simple sugars, which possibly influence food value;
- d) Lignin and cellulose as constituents that measurably influence texture and palatability.

The scheme outlined below allows these constituents, and the ash content, which is needed to calculate results to an ash free basis and allow for soil contamination, to be conveniently determined.

2. Methods

Preparation of samples

Samples previously dried to constant weight at 80 °C were crushed in an analytical ball-mill for 2 minutes.

Ash

A sample (approx. 50 mg) was ashed at 550° for 3 hours.

Total nitrogen

The Micro-Kjeldahl method was used for samples of approx. 50 mg.

Matter extracted in petroleum ether

200–500 mg of prepared leaf material was extracted in 10 ml of petroleum ether (40–60° boiling range) in a closed flask for 24 hours, the extract filtered through a Whatman No. 1 filter paper and the residue washed with small portions of the solvent. The filtrate and washings were transferred to a small beaker, evaporated in a stream of air, dried in a vacuum desiccator over paraffin wax, and weighed.

Matter extracted in 50% aqueous methanol

Petrol-extracted leaf material was air-dried and extracted with 10 ml of 50% aqueous methanol in a closed flask for 24 hours. The extract, usually yellow or pale brown, was filtered through a Whatman No. 1 filter paper into 100 ml volumetric flask, and the residue washed, first with a little water, then with methanol. This procedure effectively removes residual polyphenols and sugars. The combined extract was diluted to 100 ml with water, so that not more than 15 ml methanol remained in the extract, which is not enough to interfere with the subsequent determinations.

Polyphenols

Leaf extracts contain complex mixtures of phenolic components so that the total polyphenol content can neither be measured absolutely, nor related to any one phenolic compound. The relative reducing capacity of the extractable polyphenols was measured using the Folin-Denis reagent. Other leaf components do not interfere. KING and BLOOMFIELD (1966) found PRO's (1952) modification of the Folin-Denis method to be suitable for aqueous leaf extracts, and we used this method.

5 ml of Folin-Denis reagent is added to a suitable aliquot of the 50% aqueous methanol extract in a 100 ml volumetric flask. After 3 minutes, 10 ml of saturated aqueous sodium carbonate are added and the mixture diluted to 100 ml with water. The solution is shaken occasionally during the next hour, filtered if necessary and the optical density of the

resulting blue solution measured at 725 μ m, using a Hilger Spekker absorptionmeter, with a No. 607 filter and 1 cm cell, against a reagent blank. Polyphenols are conveniently expressed as Spekker drum difference units $\times 10^{-3}$ per mg ash-free oven-dry leaf material.

Sugars

Total sugar was determined with Morris's anthrone reagent (1948) and the result expressed as glucose, the predominant sugar in oak and beech leaf litter; sucrose and rhamnose also occur in small quantities but their response to the anthrone reagent is sufficiently close to that of glucose to cause little error.

5 ml of the suitably-diluted test solution is added to 10 ml of anthrone reagent; after 15 minutes the absorbance was measured colorimetrically (Spekker, No. 607 filter, 1 cm cell), and the results calculated to give the percentage of glucose in the ashfree, oven-dry leaf. The relationship between glucose concentration and absorbance was linear, from 0—200 μ g glucose. It is important to use the same relative volumes of test solution and anthrone reagent.

Lignin (BRAUNS, 1952)

72% W/W Sulphuric acid (15 ml) is added to the air-dry, petroleum ether and aqueous methanol-extracted leaf material. The mixture is occasionally stirred during 2 hours and is then washed into a 750 ml conical flask with 100 ml water. The flask is heated on a boiling water bath for 4 hours and the lignin residue filtered on a weighed glass filter crucible, porosity 3. The lignin is washed successively with hot water, ethanol and ether, and dried to constant weight at 105°. The ash content of the residue is determined and the lignin content calculated as the percentage in the oven-dry ash-free leaf.

Holocellulose (JERMYN, 1955)

As for lignin, cellulose is determined on the residue from the petroleum ether and aqueous methanol extract. The extracted leaf material is treated with sodium chloride in dilute acetic and the residual lignin and ash in the crude cellulose residue is determined as described above.

3. Results and discussion

Table 1 shows the reproducibility of the methods, the results given are for duplicate samples.

Table 1 Chemical analysis of leaf material

Leaf Type	Ash	Total N	Soluble in petroleum ether.	Soluble in 50% methanol.	Polyphenols*)	Sugars	Lignin	Cellulose**)
%	%	%	%	%		%	%	%
HARD	1.38	1.21	1.77	8.9	7.2×10^{-2}	2.69	38.5	44.6
	1.05	1.19	1.79	10.6	7.8×10^{-2}	2.90	37.9	—
MEDIUM	1.75	1.47	0.39	9.5	7.7×10^{-2}	2.39	36.1	46.3
	1.84	1.47	1.01	9.0	7.6×10^{-2}	2.65	35.3	—
SOFT	2.47	1.75	0.99	10.0	4.1×10^{-2}	0.94	35.5	50.9
	2.22	1.77	1.08	11.0	3.9×10^{-2}	0.66	35.4	—

*) Polyphenols were measured with a Hilger Spekker Colorimetric absorptionmeter and the results are expressed in arbitrary units of "instrument drum difference/mg oven-dry leaf".

**) Cellulose determinations on one leaf sample only.

Leaves of oven-dried autumn-fallen oak (1964) were graded into hard, medium and soft types (HEATH and ARNOLD, 1966), then analysed using 500—600 mg of material. Results are expressed on the basis of leaves oven-dried after exposure and sampling.

The complex composition and variability of leaves makes it difficult to correlate their chemical properties with their acceptability to soil animals, compounds vary in amount between both hard and soft leaves and between individual leaves of any class. Grading leaves by hand and eye for hardness or softness is subjective, and replication cannot completely overcome the problem set by leaves differing in composition. Although there seem to be quantitative differences between the soluble components of hard and soft leaves, qualitative differences were not detected; examination by paper chromatography of the polyphenols and sugars in simultaneously gathered hard and soft oak leaves showed

Table 2 Analysis of field replicates of hard and soft beech leaves exposed in litter layer to soil fauna*)

No. of weeks exposed in litter layer	Leaf type	Soluble in petroleum ether %	Polyphenols D.D. mg O D Leaf $\times 10^{-3}$	Sugars %	Lignin %	Total N %	Soluble in 50% Methanol %
4	HARD						
	BAH (1)**)	0.88	9.87	1.77	43.0	1.32	3.53
	BAH (2)	0.82	9.17	2.48	42.2	1.29	6.03
	BAH (3)	0.94	7.97	2.29	43.1	1.58	5.11
4	BAH (4)	0.74	10.1	2.23	44.1	1.25	3.63
	SOFT						
	BAS (1)	1.05	6.51	2.41	54.3	1.73	4.08
	BAS (2)	0.84	6.81	2.35	41.3	1.55	4.67
8	BAS (3)	0.49	5.30	1.80	35.8	1.56	6.66
	BAS (4)	0.28	6.36	2.27	41.3	1.62	7.88
	HARD						
	BAH (1)	0.34	7.88	3.18	44.2	1.55	6.42
8	BAH (2)	0.58	8.17	2.54	41.3	1.37	5.88
	BAH (3)	1.14	7.52	2.85	47.5	1.59	6.80
	BAH (4)	0.57	8.43	3.08	46.2	1.31	6.37
	SOFT						
12	BAS (1)	0.62	4.07	2.00	43.7	1.81	4.71
	BAS (2)	1.34	3.40	1.36	37.3	1.59	4.41
	BAS (3)	1.09	3.37	1.78	43.7	1.71	4.10
	BAS (4)	0.84	3.60	1.33	42.6	1.61	3.38
16	HARD						
	BBH (1)	0.31	7.60	2.53	48.0	1.70	6.27
	BBH (2)	0.93	7.18	2.37	49.4	1.79	4.72
	BBH (3)	0.38	6.37	2.17	47.6	1.60	5.99
16	HARD						
	BAH (1)	0.52	4.57	2.13	55.1	1.79	4.51
	BAH (2)	0.72	5.37	2.21	49.3	1.74	5.77
	BAH (3)	0.12	5.50	2.45	48.8	1.93	6.98
16	BAH (4)	0.43	4.91	2.38	43.2	1.80	6.08
	SOFT						
	BAS (1)	1.43	4.57	5.08	52.5	2.25	7.62
	BAS (2)	0.66	3.43	1.37	33.7	1.76	6.00
16	BAS (3)	0.12	4.56	2.13	52.3	1.83	5.60
	BAS (4)	1.55	4.45	1.81	46.7	1.91	4.58

*) See HEATH and ARNOLD 1966, paper II of this series.

**) The numbers (1)—(4) in the column leaf type refer to replicate batches of leaves exposed.

that the same components were present in each, but in different relative proportions (Table 1). However, qualitative differences between the composition of leaves of different plant species probably influence their relative palatability to soil animals, irrespective of thickness or hardness.

The analysis of autumn-fallen oak leaves (1964), not exposed to soil animals, and graded subjectively into hard and soft categories, showed significant differences between polyphenol, total nitrogen, lignin, cellulose and sugar contents (Table 1).

Tables 2 and 3 show that after exposure to soil fauna more polyphenols are consistently retained by hard than soft leaves; total nitrogen was consistently less in the hard leaves throughout the exposure time. The lignin contents seem not to differ until after 8 weeks, whereas the sugar contents changed. Table 4 shows that the extent of animal feeding, i. e. how much disappeared is inversely proportional to the polyphenol content, and recent experiments we have made with time lapse cinematography indicate that earthworms prefer leaves that have been extracted with 50 % methanol and petroleum ether to untreated leaves. This seems to indicate that the polyphenol and/or the wax content may determine palatability and taste, and hence why hard and soft leaves disappear at very different rates, especially when exposed to earthworms.

Table 3 Means of the treatments

No. of weeks exposed in litter layer	Leaf type	Soluble in petroleum ether %	Polyphenols D.D./mg O D Leaf $\times 10^{-2}$	Sugars %	Lignin %	Total N %	Soluble in 50 % Methanol %
4	HARD	0.84	9.28	2.19	43.1	1.36	4.58
	SOFT	0.67	6.25	2.21	43.2	1.61	5.82
8	HARD	0.37	8.00	2.91	44.8	1.46	6.37
	SOFT	0.97	3.61	1.62	41.8	1.68	4.15
12	HARD	0.54	7.05	2.36	48.3	1.70	5.66
16	HARD	0.45	5.09	2.29	49.1	1.87	5.84
	SOFT	0.94	4.27	3.10	46.3	1.94	5.95

Table 4 Percentage disappearance as estimated photometrically (HEATH & ARNOLD 1966) of the discs in the above treatments

No. of weeks exposed in litter layer	AH	AS	BH	BS
4	0.8	5.3	0.8	3.8
8	0	6.0	6.0	9.0
12	10.0	10.8	35.0	100
16	6.5	10.8	48.0	100

4. Literature

- BRAUNS, F. E., 1952. Tappi Standard method T 13 for the determination of lignin; in "The Chemistry of Lignin", Academic Press, Inc., New York, 176, 177.
- GILBERT, O., and K. L. BOCKOCK, 1960. Changes in leaf litter when placed on the surface of soils with contrasting humus types. II. Changes in the nitrogen content of oak and ash litter. *J. Soil Sci.*, 11, 10—19.
- HEATH, G. W., 1962. Biology of forest soils. Rep. Forest Res., 96, 97.
- and C. A. EDWARDS, and M. K. ARNOLD, 1964a. Some methods of assessing the activity of soil animals in the breakdown of leaves. *Pedobiologia*, 4, 80—87.
- and H. G. C. KING, 1964b. The palatability of litter to soil fauna. VIIIth Int. Congr. Soil Sci., Bucharest. In the press.
- and M. K. ARNOLD, 1966. Studies in leaf-litter breakdown. II. Breakdown rate of "sun" and "shade" leaves. *Pedobiologia*, 6, 238—243.

- JERMYN, M. A., 1955. Cellulose and hemicellulose; in „Modern Methods of Plant Analysis”, ed. M. V. TRACEY and K. PAECH, Springer-Verlag, Berlin, 2, 197—225.
- KING, H. G. C., and C. BLOOMFIELD, 1966. The reaction between water soluble tree leaf constituents and ferric oxide in relation to podzolisation. *J. Sci. Fd. Agric.* 17, 39—43.
- MORRIS, D. L., 1948. The quantitative determination of carbohydrates with Dreywood's anthrone reagent. *Science*, 107, 254—225.
- PRO, M. J., 1952. Report on spectrophotometric determination of tannin in wines and whiskies. *J. Ass. off. agric. Chem.*, 35, 255—257.
- SOKOLOV, D. F., 1960. The decomposition and mineralisation of fallen oak and maple leaves under dry steppe conditions. *Bjull. Mosk. obsc. ispyt. prirody*, 65 (2), 67—84.

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